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4-HYDROPEROXYCYCLOPHOSPHAMIDE INHIBITS PROLIFERATION BY HUMAN GRANULOCYTE-MACROPHAGE COLONY-FORMING CELLS (GM-CFC) BUT SPARES MORE PRIMITIVE PROGENITOR CELLS

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Abstract—Despite its considerable toxicity to haemopoietic colony-forming cells, 4-hydroperoxycyclophosphamide (4-HC) has successfully been used to purge marrow of leukaemic cells before it is used to rescue patients from high-dose chemoradiotherapy. These conflicting observations indicate that haemopoietic progenitor cells that are not detected by the established colony-forming assays survive exposure to 4-HC and repopulate the marrow. The recent finding that murine spleen colony-forming cells (CFU-S) are resistant to 4-HC [Porcellini A, *et al.* (1983) *Expl Hemat.* **11** (suppl 14) 331 (abstract)] [14] also indicates that sensitivity to 4-HC can be used to distinguish primitive progenitor cells from committed progenitor cells. As part of a study on the nature of a population of blast colony-forming cells in human bone marrow, we tested their sensitivity to 4-HC to see whether they also are spared by the drug. We found that 4-HC had much less effect on the blast colony-forming cells than on the granulocyte-macrophage colony-forming cells (GM-CFC). This result suggests that the blast-colony-forming cells may be early human haemopoietic progenitor cells.

Key words: 4-Hydroperoxycyclophosphamide, leukaemia, autologous marrow transplant, stem cells.

INTRODUCTION

Two derivatives of cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC) and ASTA-Z-7557, have been used to deplete marrow of leukaemic cells so that it can more safely be used to rescue patients from myeloablative therapy [8–10, 12, 13, 18]. This approach is based on the finding that rat bone marrow can be 'purged' of contaminating leukaemic cells by incubation with 4-HC [17].

The effects of 4-HC and ASTA-Z-7557 on normal and leukaemic clonogenic haemopoietic progenitor cells have been investigated to test the assumption that these drugs selectively kill the malignant cells. However, it has been shown that 4-HC and ASTA-Z-7557 are toxic to normal colony-forming cells *in vitro* and that they do not exert any selective effect on clonogenic human leukaemia cells [1, 7, 11, 13, 14, 16, 18].

Abbreviations: BFU-E, erythroid burst-forming unit; *CFU-S,* spleen colony-forming unit; *FCS,* fetal calf serum; *GEMM-CFC,* multipotent colony-forming cells; *GM-CFC,* granulocyte-macrophage colony-forming cells; *4-HC,* 4-hydroperoxycyclophosphamide; *MP,* methylprednisolone; *PHA-LCM,* phytohaemagglutinin-stimulated leucocyte-conditioned medium.

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In contrast to the committed haemopoietic progenitors that form colonies in semi-solid cultures, murine spleen colony-forming cells (CFU-S) are spared by 4-HC and ASTA-Z-7557 [14]. The purpose of this study was to test the effects of 4-HC on a population of blast colony-forming cells in human bone marrow [3–6] to determine whether they resemble murine CFU-S or committed progenitor cells in their sensitivity to the drug.

MATERIALS AND METHODS

Bone marrow cells were obtained, with informed consent, from donors of marrow for transplantation into patients with aplastic anaemia or leukaemia.

Blast colony assay

Preparation of stromal feeder layers: the feeder layers were prepared in the 35 mm petri dishes to be used for the assay. Mononuclear cells (5×10^5) obtained from normal human bone marrow by centrifugation over Lymphoprep (Nyegaard, Oslo) were incubated (37°C in humidified CO_2 in air) in 1 ml of α-medium (GIBCO) containing 15% fetal calf serum (FCS; GIBCO) and 1.7×10^{-6} M methylprednisolone (MP; Solumedrone, Upjohn). The cells adhering to the dish were fed at weekly intervals by complete replacement of the medium, FCS and MP until a confluent layer of fibroblasts

and fat cells had formed. Confluence was usually achieved after 4–6 weeks.

Addition of marrow for assay: Normal human bone marrow mononuclear cells were diluted to 10^6 cells per ml in α-medium supplemented with 15% FCS and depleted of adherent cells by incubation in plastic tissue culture flasks for 2 h at 37°C in 7.5% CO_2 in air. The non-adherent fraction from 5×10^5 mononuclear cells in 1 ml α-medium + 15% FCS was then added to each of the established feeder layers and incubated for a further 2 h at 37°C in 7.5% CO_2 in air. The feeder layers were washed three times to remove any cells that had not attached to the stromal cells and the medium was replaced by 1 ml of 0.3% agar in α-medium + 15% FCS. The plates were incubated for 5 days at 37°C in humidified 7.5% CO_2 in air.

Evaluation of colony formation: All colonies of more than 20 cells were counted before the plates were dehydrated and stained with May-Grünwald and Giemsa [2]. The colonies were then classified morphologically (Fig. 1). Type I colonies consisted of uniform populations of blast cells with no apparent features of maturation; type II colonies contain heavily granulated cells, all of which are of similar appearance; type III colonies are indistinguishable from the granulocyte colonies produced by GM-CFC in agar culture.

GM-CFC assay

The non-adherent fraction of 10^5 mononuclear bone marrow cells from the same cell suspension as that used for the blast colony assay was plated in a 1 ml semi-solid culture containing 0.3% agar and 10% medium conditioned by phytohaemagglutinin-stimulated blood mononuclear cells (PHA-LCM) in α-medium + 15% FCS. The plates were incubated at 37°C in humidified 7.5% CO_2 in air and the resulting colonies were counted once after 7 days and again after 14 days of incubation.

Treatment with 4-HC

Non-adherent bone marrow cells (1×10^6/ml) were incubated with 4-HC (freshly dissolved in calcium and magnesium free phosphate buffered saline) at concentrations between 0 and 50 μg/ml for 30 min at 37°C. The cells were placed on ice for 5 min and then washed with cold medium before they were plated in the colony culture systems.

RESULTS

Cultures of normal marrow produce 24 ± 4 (mean ± S.E.M.; $n = 21$) colonies per 10^5 mononuclear cells of which 66 ± 10% are type I and 32 ± 10% are type II (see Methods).

The survival of the type I blast colony-forming cells and of GM-CFC following treatment *in vitro* with 4-HC are given in Fig. 2. The day 14 GM-CFC and the day 7 GM-CFC were considerably more sensitive to 4-HC than were the type I blast colony-forming cells.

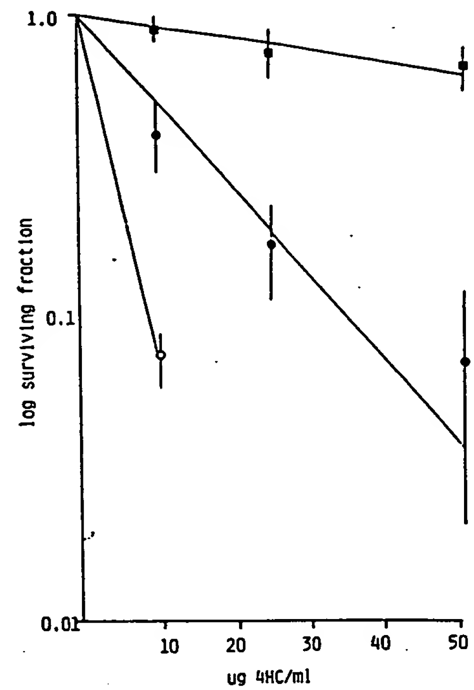

FIG. 2. The effects of in vitro treatment with 4-HC on subsequent colony formation by ■ type I blast colony-forming cells, ● day 7 GM-CFC and ○ day 14 GM-CFC. Each experimental point is the mean of five replicate experiments; the bars represent 1 S.E.M.

DISCUSSION

The introduction of 4-HC and ASTA-Z-7557 into clinical practice has led to tests of their effects on normal haemopoietic and leukaemic colony-forming cells. It is now well established that the drugs are toxic to human GM-CFC, BFU-E and GEMM-CFC as well as to leukaemic colony-forming cells [1, 7, 11, 13, 15, 16, 18]. Thus, comparison of the *in vitro* results with the performance of marrow reinfused after treatment with 4-HC shows clearly that the current assays for haemopoietic progenitor cells do not adequately predict the capacity of the marrow to engraft.

In addition to the clinical evidence, the finding that murine stem cells (CFU-S) are spared by 4-HC [14] indicates that resistance to the drug is a property of primitive haemopoietic progenitor cells. The blast colony-forming cells investigated here are also relatively resistant to 4-HC. There are other grounds for suggesting that the blast colony-forming cells belong to a primitive haemopoietic progenitor cell population. They are slowly cycling cells; they do not express HLA-DR (Ia-like) surface antigens; they are capable of self-renewal in culture and they can be separated from other colony-forming cells, including GEMM-CFC by 'panning' on cultured stromal layers [3–6]. However, it will be necessary to extend these preliminary studies to higher, clinically used doses of 4-HC before the results can be used to suggest that the blast colony-forming cells may provide a better indication than the other colony assays of the transplantability of human marrow after it has been manipulated *in vitro*.

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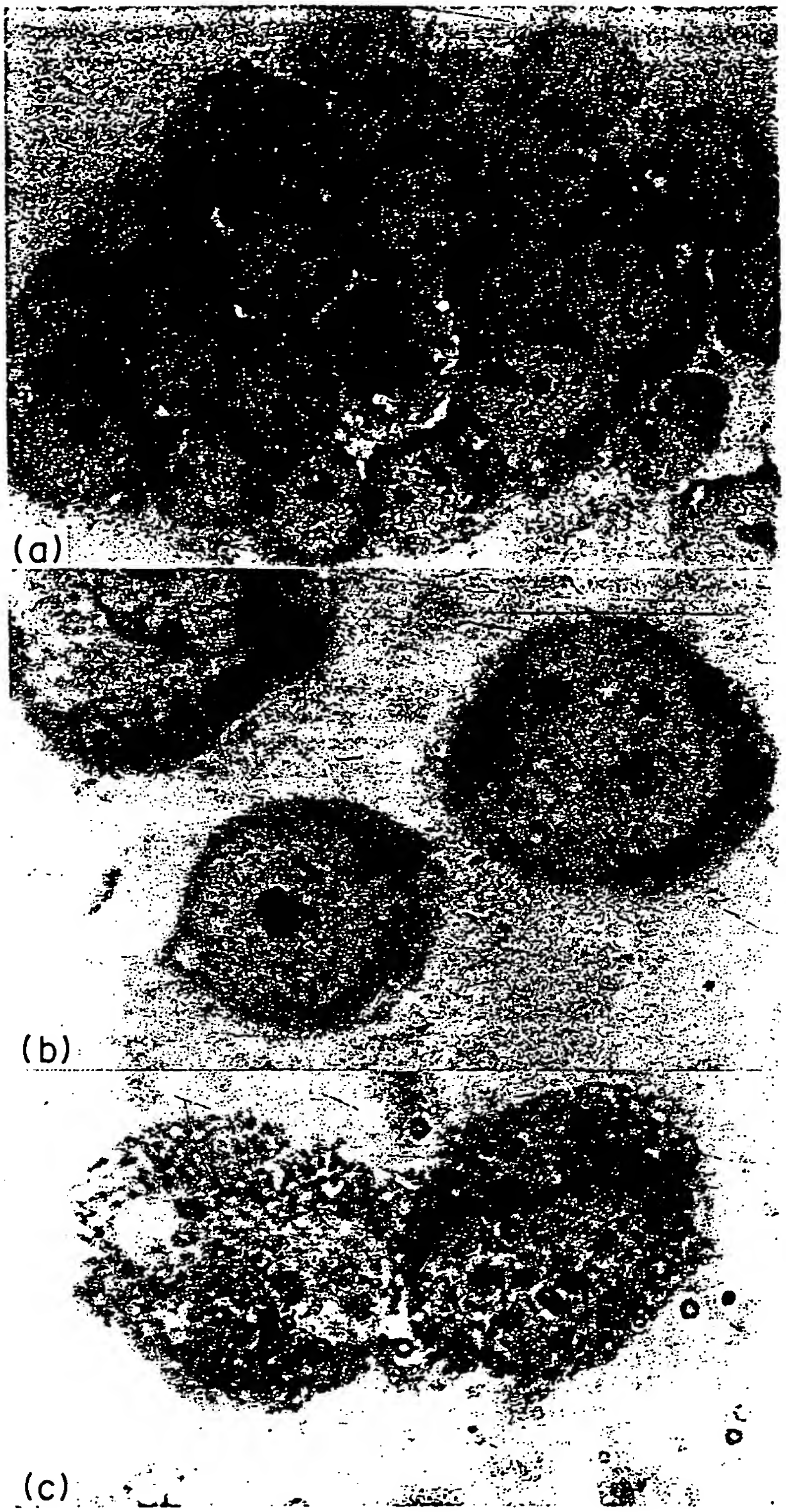

FIG. 1. (a) Appearance of a type I blast colony (× 800). (b) Cells from a type I blast colony (× 3200). (c) Cells from a type II blast colony (× 3200).

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